

In re Application 10/500,784
Response to Office Action dated Feb 23, 2007
Page 3 of 5

RECEIVED
CENTRAL FAX CENTER
JUN 25 2007

REMARKS

Applicants have amended claim 37 and claim 39 to more particularly and distinctly claim their invention. Applicants add new claims 43 and 44. Support for the amendment of claim 37 may be found at Para [0128] and examples of such antibodies are identified in Examples 5 and 6. Support for new claims 43 and 44 may be found in original claim 7 and Para [0064] to Para [0067]. No new matter has been introduced by these amendments.

I. Rejection Under 35 U.S.C. § 101

A. Claim 37 has been rejected for being directed to non-statutory subject matter. Applicants have amended claim 37 to recite "isolated" antibody obviating this rejection.

B. Claims 37-39 have been rejected as lacking a substantial "real world" utility. The Office asserts that because there is no function assigned to SEQ ID NO 2. Applicants respectfully traverse this rejection.

As noted in the Utility Guidelines issued by the Office, a compound need only have one utility. Consistently, throughout the examples the guidelines indicate that no §101 should be made where there is a specific substantial utility. The fact that an antibody specifically binds to an identified receptor found on the surface of mast cells and mast cells are well known to be associated with allergic diseases, such as asthma, establishes a "real world" credible utility. The antibody can be used to identify mast cells in lung tissue as shown in Example 6. Applicants also enclose a paper published in Genomics 86:68-75 (2005) showing experiments further characterizing the antibodies of the present invention.

Even if the Office does not accept the "real world" correlation, Claims 37-39 should not be rejected. For example, in Example 8 "Therapeutics not Associated with a Disease", the guidelines indicate that the compound itself should not be rejected even if the method of treatment utility was not established (Example enclosed for the Examiner's convenience). Applicants disagree with the Office that treatment of mast cell related diseases is not sufficiently correlative, but the method claims are not being examined at this time. Given the statement in the guidelines of Example 8 that the compound itself should not be rejected under §101, Applicants request that the rejection of claims 37-39 be withdrawn.

In re Application 10/500,784
Response to Office Action dated Feb 23, 2007
Page 4 of 5

II. Rejection Under 35 U.S.C. § 112, First Paragraph

Claims 37-39 have been rejected §112, First Paragraph, because of the §101 rejection above. Applicants respectfully traverse this rejection.

Applicants submit that the antibodies of the present invention are fully enabled and have a substantial real world utility as discussed above. Applicants have shown how to make and use the invention and sufficient disclose the region of the receptor to which these antibodies bind. Therefore, Applicants request that this rejection be withdrawn.

III. Rejection Under 35 U.S.C. § 112, Second Paragraph

Claim 39 has been rejected as being indefinite for failing to particularly and distinctly claim that which Applicants regard as their invention. Applicants have amended claim 39 and added claims 43 and 44 rendering this rejection moot. Applicants request that the rejection be withdrawn.

IV. Rejection Under 35 U.S.C. § 102

Claims 37-39 have been rejected as being anticipated by WO98/30582. The Office asserts that the reference teaches antibodies that bind to EH203_2 (Reference SEQ ID NO 27). Applicants respectfully traverse this rejection in view of the amendment to claim 37.

In order for a reference to anticipate the claimed invention, it must teach all of the limitations of the claim. WO98/30582 does not teach antibodies that specifically bind to amino acid residues 105-187. Therefore, Applicants request that the rejection be withdrawn.

V. Rejection Under 35 U.S.C. § 103(a)

A. Claim 37 has been rejected as being unpatentable over WO98/30582 in view of Harlow et al. Applicants respectfully traverse this rejection in view of the amendment to claim 37. As noted in Section IV above, WO98/30582 does not teach antibodies that specifically bind to amino acid residues 105-187. Harlow does not overcome this deficiency. Therefore, Applicants request that the rejection be withdrawn.

B. Claims 37-39 have been rejected as being unpatentable over WO98/30582 in view of U.S. Pat. No. 6,108,370. Applicants respectfully traverse this rejection in view of the amendment to claim 37. As noted in Section IV above, WO98/30582 does not teach antibodies that specifically bind to amino acid residues

In re Application 10/500,784
Response to Office Action dated Feb 23, 2007
Page 5 of 5

105-187. The '370 patent does not overcome this deficiency. Therefore, Applicants request that the rejection be withdrawn.

C. Claims 37-39 have been rejected as being unpatentable over WO98/30582 in view of WO96/34096. Applicants respectfully traverse this rejection in view of the amendment to claim 37. As noted in Section IV above, WO98/30582 does not teach antibodies that specifically bind to amino acid residues 105-187. The WO96/34096 application does not overcome this deficiency. Therefore, Applicants request that the rejection be withdrawn.

D. Claims 37-39 have been rejected as being unpatentable over WO98/30582 in view of U.S. Pat. No. 6,132,729. Applicants respectfully traverse this rejection in view of the amendment to claim 37. As noted in Section IV above, WO98/30582 does not teach antibodies that specifically bind to amino acid residues 105-187. The '729 patent does not overcome this deficiency. Therefore, Applicants request that the rejection be withdrawn.

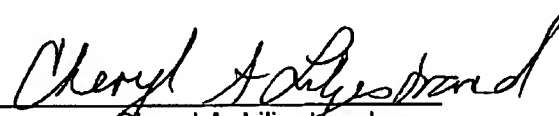
CONCLUSION

In view of the amendments and remarks, Applicants submit that the present application is in condition for allowance and request rejoinder of claims 40-42.

Respectfully Submitted,

DATE: June 25, 2007.

BY:


Cheryl A. Liljestrand
Reg. No. 45,275

RECEIVED
CENTRAL FAX CENTER

JUN 25 2007

Example 8: "Therapeutics" Not Associated with a Disease

Specification: Compound A is disclosed to inhibit enzyme XYZ, a well-known enzyme which is a member of the family of tyrosine kinases, *in vitro*. The specification states that compound A can be used to treat diseases caused or exacerbated by increased activity of enzyme XYZ. No actual diseases are named.

Claims:

1. Compound A.
2. A method of treating a disease caused or exacerbated by increased activity of enzyme XYZ consisting of administering an effective amount of compound A to a patient.

Analysis: The following analysis includes the questions that need to be asked according to the guidelines and the answers to those questions based on the above facts:

1) Based on the record, is there a "well established utility" for the claimed invention? With respect to claim 2, since the claim is directed to a specific method of use, the utility of this claim is limited to that use and the examiner should not look to a "well established utility" for the composition used in the claimed method. Consequently, the answer to the question is no for claim 2. With respect to claim 1, the answer is different. Enzymes catalyze certain reactions involving the enzyme substrate. Here, since enzyme XYZ is a well-known tyrosine kinase, the substrate for the enzyme and the reaction which the enzyme catalyzes must also be well known.

Since all of this is well known it is reasonable to infer that an inhibitor of enzyme XYZ, such as compound A, would have a "well-established utility" in controlling the enzyme/substrate interaction in the known reaction. Therefore, compound A has a "well established utility", no rejection under 35 U.S.C. § 101 should be made against claim 1, and there is no need to go further in the analysis with respect to claim 1.

2) Has the applicant made any assertion of utility for the specifically claimed invention? The answer is yes. Claim 2 has the asserted utility of treating a disease caused or exacerbated by increased activity of enzyme XYZ.

3) Is the asserted utility specific? In this case, the specification teaches that the claimed compound inhibits a particular enzyme (XYZ). Therefore, compound A has properties and uses that are not applicable to a general class of compounds. Therefore, the answer is that the invention of claim 2 has a specific utility.

4) Is the asserted utility substantial? Since neither the specification nor the art of record disclose any diseases or conditions caused or exacerbated by enzyme XYZ, the asserted utility in this case essentially is a method of treating an unspecified, undisclosed disease or condition, which does not define a "real world" context of use. Treating an unspecified, undisclosed disease or condition would require or constitute carrying out further research to identify or reasonably confirm a "real world" context of use. Therefore, the answer to this question is no with respect to claim 2.

Therefore no rejection under 35 U.S.C. § 101 should be made against claim 1 but both a 35 U.S.C. § 101, as well as 35 U.S.C. § 112, first paragraph, utility rejection should be made against claim 2.

Once the rejection has been made with respect to claim 2, the applicant bears the burden of rebutting it. Upon receiving applicant's response, the examiner should review the original disclosure, any evidence relied upon in establishing the utility rejections under 35 U.S.C. § 101 and 35 U.S.C. § 112, first paragraph, any amendments and any new reasoning or evidence provided by the applicant in support of the asserted utility.

The following situations are most probable:

(1) Applicant provides a reference, published before the filing date of the application, which teaches that certain diseases are associated with increased activity of enzyme XYZ. In this case the examiner should withdraw the utility rejections under 35 U.S.C. § 101 and 35 U.S.C. § 112, first paragraph, for claim 2.

(2) Applicant submits an opinion declaration under 37 C.F.R. 1.132 by a qualified person of skill in the art which states that specific disease conditions are known to the skilled artisan to be either caused or exacerbated by increased activity of enzyme XYZ. The declarant identifies specific diseases and/or conditions. After reviewing the record in its entirety, the Examiner should only maintain this rejection if evidence of more probative value than the declaration exists which establishes a basis for doubting the objective truth of the declaration. Unsupported scientific reasoning is not more probative than the declaration. If the examiner maintains the rejection,

Available online at www.sciencedirect.com

SCIENCE @ DIRECT®

Genomics 86 (2005) 68–75

GENOMICS

www.elsevier.com/locate/ygeno

Identification and expression of a new type II transmembrane protein in human mast cells[☆]

Kang Li^{a,*}, Shen-Wu Wang^a, Yucheng Li^a, Rachel E. Martin^a, Lixin Li^a, Meisheng Lu^a,
Salah-Eddine Lamhamedi-Cherradi^a, Guanghui Hu^{a,1}, Sossiena Demissie-Sanders^a,
Ji Zheng^a, Fan Chung^b, Tim Oates^b, Zhengbin Yao^a

^aTanox, Inc., 10301 Stella Link Road, Suite 110, Houston, TX 77025, USA^bNational Heart & Lung Institute, Imperial College, London, UK

Received 26 August 2004; accepted 16 March 2005

Available online 15 April 2005

Abstract

A cDNA encoding a new type II transmembrane protein has been isolated from human mast cells by subtraction cloning. This cDNA contains an open reading frame of 186 amino acids. RT-PCR analysis showed that this gene is differentially expressed in mast cells. Therefore, the peptide encoded by this gene was termed mast cell-expressed membrane protein 1 (MCEMP1). The MCEMP1 gene contains seven exons and was mapped to human chromosome 19p13.3. The epitope-tagged MCEMP1 has been expressed in mammalian cells and found to be localized to the cellular membrane with its C-terminus extending to the outside of the membrane and N-terminus into the cytoplasmic compartment. Monoclonal antibodies against MCEMP1 were generated and characterized by immunoprecipitation and FACS. The results showed that the native MCEMP1 is expressed in cord blood-derived mast cells and HMC-1 and THP-1 cell lines, but not in other cell types that we have tested. Immunohistochemical staining of human lung sections showed that MCEMP1 staining is specifically associated with lung mast cells.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Mast cell; Transmembrane protein; Expression; Lung; Cellular marker

Mast cells are major immune effector cells in the human defense system against parasite infection. These granular immune cells originate from hematopoietic stem cells in the bone marrow and complete their development only after they migrate into diverse peripheral tissues [1–3]. Mature mast cells express the high-affinity IgE receptor FcεRI on their surface and can be activated by IgE cross-linked with

specific antigens or allergens. Mast cells may also be activated via IgE-independent mechanisms [4,5]. Complement factors, C3a, C5a, neuropeptides, and calcium ionophores such as A23187 have been shown to activate mast cells in vivo and in vitro, respectively [6]. Mast cells exert their functional effects by releasing a wide variety of preformed secretory inflammatory mediators such as histamine, tryptase, and neutrophil chemotactic factor as well as newly synthesized cytokines including TNFα, IL-4, IL-13, IL-5, IL-10, and chemokines [7–9].

It is well known that human mast cells play critical roles in the pathogenesis of many allergic and inflammatory diseases, such as asthma, allergic rhinitis, anaphylaxis, and atopic dermatitis. The mast cells are responsible for most of the early events in allergic reactions through the release of cytokines and other mechanisms that contribute to the expression of late-phase reactions and chronic allergic inflammation [7–9].

Abbreviations: MCEMP1, mast cell-expressed membrane protein 1; CBMC, cord blood-derived mast cell; mAb, monoclonal antibody; RT-PCR, reverse transcriptase-polymerase chain reaction.

* Sequence data from this article have been deposited with the GenBank Data Library under Accession Nos. AF461155 and AF461156.

* Corresponding author. Fax: +1 713 578 5046.

E-mail address: kli@tanox.com (K. Li).

¹ Present address: GlaxoSmith Kline, 709 Swedeland Road, King of Prussia, PA 19406, USA.

0888-7543/\$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygeno.2005.03.006

Mast cells have also been observed to be involved in a multitude of neoplastic, fibrotic, and inflammatory processes such as lymphoproliferative disorders, interstitial lung disease, multiple sclerosis [10,11], and rheumatoid arthritis [12–15]. The number of mast cells is highly elevated in certain inflammatory diseases, such as inflammatory bowel disease [16]. Recent studies suggest that mast cells may play a role in the progression of heart failure [17]. Not only are mast cells found in human heart but their number and density are also increased in the failing heart and in ischemic cardiomyopathy patients [17].

The transmembrane proteins on the mast cell surface play a crucial role in the contact with cells in the peripheral tissues and in mediating regulatory signals from their environment. In this study, we report the identification and characterization of a new mast cell-expressed membrane protein, MCEMP1.

Results

Identification and cloning of MCEMP1 cDNA

MCEMP1 cDNA was identified by cDNA subtractive hybridization using human mast cell mRNA as a tester and a pool of mixed mRNAs from human Daudi, THP-1, and TF-1 cell lines as a driver. A partial cDNA clone initially isolated by the subtractive hybridization matched only to a number of EST clones (GenBank Accession Nos. BM537757, BM922263, B1818132, AA482321, CD640454) that contain partial cDNA sequences, but it showed no significant homology to any cDNA sequences that encode known or predicted proteins in the GenBank database. Two oligonucleotide primers were designed based on the partial cDNA sequence and used to screen a human leukocyte cDNA array library (OriGene, Rockville, MD, USA) to clone the full-length cDNA. Several cDNA clones have been isolated and one such cDNA clone (9E) contains a bona fide full-length coding region, 450 bp of 5' untranslated sequence, and 726 bp of 3' untranslated sequence. The longest open reading frame is predicted to encode a protein of 186 aa with a calculated molecular weight of 21.2 kDa (Fig. 1A). The initiation methionine codon is defined by several criteria: (1) no methionine codon precedes in the 5' untranslated region, (2) a perfect Kozak motif [18] co-resides with the starting methionine codon, and (3) several in-frame stop codons exist upstream of the predicted initiation methionine. A single transmembrane domain was identified to extend from aa 83 to 105, but no signal peptide sequence was found in MCEMP1 using a hidden Markov model-based transmembrane helix prediction method [19].

Gene organization, alternative splicing, chromosome localization, and polymorphism

MCEMP1 cDNA was mapped to contig AC008763 on chromosome 19p13.3 by a similarity search [20]. The

alignment of MCEMP1 cDNA with the genomic DNA revealed seven exons and six introns that span ~3.2 kb (Fig. 1B). Two alternatively spliced forms of mRNAs identified during cDNA cloning were also mapped to the same region (Fig. 1B). However, the alternatively spliced forms encode only truncated proteins, since putative translation products would be prematurely aborted by stop codons in all three reading frames. A single-nucleotide polymorphism was found at codon 167 (ATT/GTT) in the cDNA sequence (9E) we obtained, as well as in the EST sequences from the GenBank database, which causes an amino acid change (Ile/Val) in the translated protein sequences. The 5' flanking sequence analysis [21] revealed a number of transcription factor binding motifs such as a classical TATA box and NF- κ B/Rel and NF-AT (nuclear factor of activated T cells) binding sites, suggesting that the 5' flanking region is a transcription-regulatory promoter.

Differential expression of MCEMP1 mRNA

The MCEMP1 mRNA levels were accessed by RT-PCR and compared among multiple human tissues (Fig. 2A) and among hematopoietic cells and cell lines (Fig. 2B). Mast cells express the highest level of MCEMP1 mRNA among all types of cell lines and primary blood cells assessed (Fig. 2B). However, in the first week of culture of cord-blood-derived CD34⁺ progenitor cells (BioWhittaker, Inc., Walkersville, MD, USA), the MCEMP1 mRNA was almost undetectable. Maximum levels of expression were reached after the primary mast cells were cultured for 5 weeks with about 35% of the cell population staining positively by anti-tryptase antibody. MCEMP1 mRNA signals were also detected in THP-1 cells, HMC-1 cells, lymphocytes, and peripheral blood mononuclear cells, but at a much lower level.

In the human tissues surveyed, MCEMP1 mRNA was detected in lung and barely detectable in trachea under the given RT-PCR conditions, but not detected in brain, heart, kidney, and liver (Fig. 2A). In light of the above results, it is expected that mast cells may be the primary cell type responsible for expression of MCEMP1 mRNA in the lung.

Expression and subcellular localization of recombinant MCEMP1

To characterize the MCEMP1 gene product, we subcloned the MCEMP1 cDNA coding region into pcDNA3.1D/V5-His vector (Invitrogen, Carlsbad, CA, USA) with a Flag tag sequence attached to the N-terminus of MCEMP1 and a V5 tag fused to the C-terminus (MCEMP1-FV, see Fig. 3A). After transient transfection into 293T cells, the tagged MCEMP1 was detected in the membrane fraction by Western blot using anti-Flag or anti-V5 antibody, confirming predictions that MCEMP1 is a transmembrane protein (data not shown).

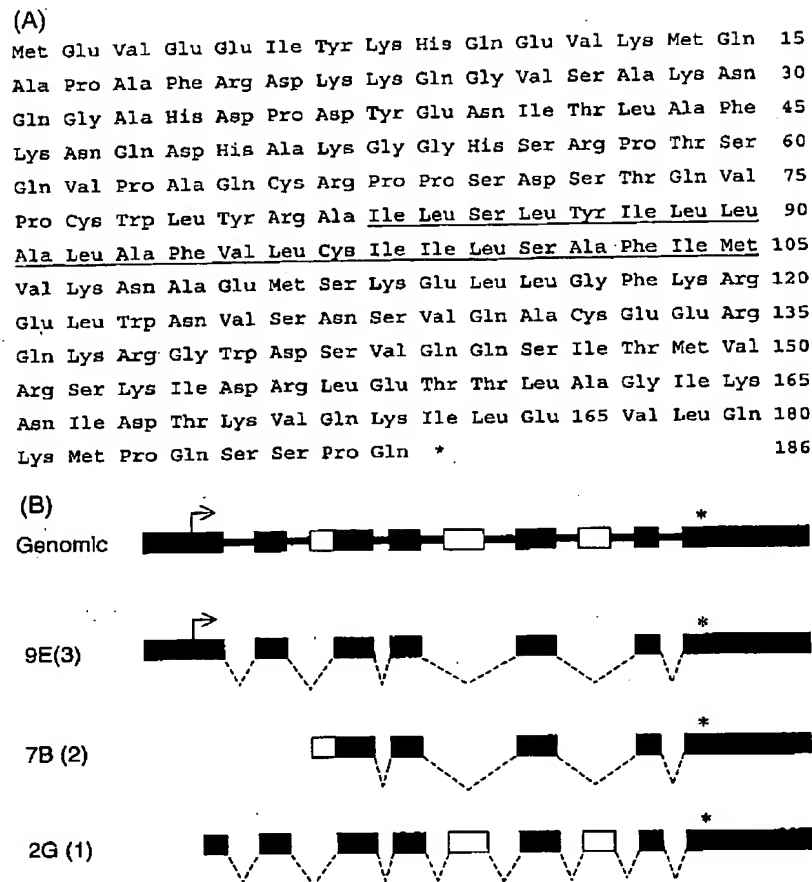


Fig. 1. (A) Amino acid sequences of MCEMP1. The predicted transmembrane region based on the hidden Markov model [19] is underlined. Asterisk indicates stop codon. (B) MCEMP1 gene structure, the authentic cDNA clone (9E), and two alternatively spliced cDNA clones (7B and 2G). The exons shared in authentic cDNA (9E) are shown as filled boxes and the exons found only in alternatively spliced forms (7B and 2G) are indicated by unfilled boxes. Introns and spliced intron regions are represented by solid lines and dash lines, respectively. The arrow indicates the starting methionine, and the asterisk represents a stop codon. The parentheses show the numbers of cDNA clones we isolated that shared the same splicing pattern as cDNA clones 9E, 7B, and 2G.

To determine further whether MCEMP1 is expressed on the cell surface and its orientation in the membrane, we incubated the MCEMP1-FV-transfected cells in vitro with FITC-conjugated anti-Flag or anti-V5 mAb. Fluorescence microscopy (Fig. 3B) and flow-cytometric analysis (not shown) showed that anti-V5 mAb binds to the MCEMP1-FV-transfected cells under both living and fixed conditions, while anti-Flag mAb binds to the MCEMP1-FV-transfected cells only after fixation (Table 1). These results indicate that MCEMP1 indeed is a transmembrane protein with the C-terminus exposed to the outside of the cellular membrane and the N-terminus to the cytoplasmic compartment.

Generation and characterization of MCEMP1 monoclonal antibodies

To investigate the expression of native MCEMP1 protein, we generated two monoclonal antibodies, MC1A8

and MC1C11. FACS analysis of living cells stained with MC1A8 showed that this antibody binds well on the cell surface of CBMC, HMC-1 cells (cell line derived from a mast cell leukemia patient) [22], THP-1 cells (cell line derived from acute monocytic leukemia tissue; ATCC), and CD14⁺ monocytes, but not to peripheral blood lymphocytes or to the cell lines derived from T cells, B cells, or epithelial cells (data not shown). Furthermore, immunoprecipitation of the membrane surface proteins using MC1A8 antibody revealed a 22-kDa protein in CBMC and HMC-1 (Fig. 4). The immunoprecipitated MCEMP1 protein band in CBMC is much stronger than that in HMC-1 cells. The protein expression profile was consistent with the mRNA expression levels in CBMC and HMC-1 cells analyzed by RT-PCR (Fig. 2). Taken together, these results indicated that monoclonal antibody MC1A8 specifically recognizes the extracellular, or the C-terminal, domain of MCEMP1. In contrast, antibody MC1C11 detected MCEMP1 protein only in Western blot or by FACS after cells were fixed (data not

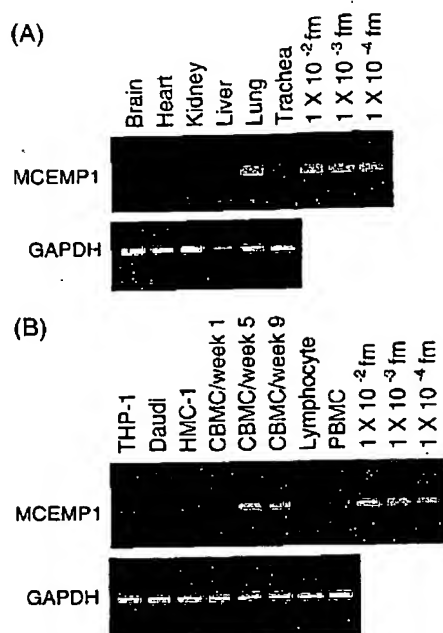


Fig. 2. (A) RT-PCR analysis of MCCEMP1 mRNA level in human tissues. The same amount (100 ng) of total tissue RNA was included in each RT-PCR. Reverse transcriptase reaction was performed using Superscriptase II kit (Invitrogen). PCR was performed using the TaKaRa *Taq* polymerase system (Sigma). Six microliters of the PCR volume (25 μ l) was loaded in each lane. The RT-PCR for MCCEMP1 (shown at the top) proceeded for 28 thermocycles and the RT-PCR for control housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; bottom) proceeded for 24 thermocycles. In the three positive control PCRs, 1×10^{-2} , 1×10^{-3} , or 1×10^{-4} fmol (fm) of pMCCEMP1-FV plasmid was put into each reaction. (B) RT-PCR analysis of human primary hematopoietic cells and cell lines. Total RNA input for each RT-PCR, positive control plasmid input, and thermocycles were the same as described for (A). Cord-blood-derived mast cells (CBMC), lymphocytes, and peripheral blood mononuclear cells (PBMC) are human primary cells. THP-1, Daudi, and HMC-1 are cell lines. For details, see Materials and methods.

shown). Our data indicate that MC1C11 may recognize only the N-terminal region after denaturation.

Localization of MCCEMP1 in mast cells of human lung tissue

Using MCCEMP1 antibody we performed immunostaining on consecutive sections of human lung cancer tissues. In the cancer-adjacent lung tissue (Fig. 5), as well as in the tumor tissue (not shown), the majority of MCCEMP1 antibody-stained cells were also positively immunostained by anti-Fc ϵ R1 α (a mast cell marker) antibody, indicating that the native MCCEMP1 is predominantly expressed in lung mast cells. The cells stained positively by both MCCEMP1 and Fc ϵ R1 α antibody accounted for about 75% of the total cells stained by MCCEMP1 antibody. A similar staining pattern was observed in the lung cancer tissue (data not shown). These results are consistent with the findings from RT-PCR analysis of MCCEMP1 mRNA.

Discussion

In this study, we have identified a cDNA encoding a new type II transmembrane protein, which is differentially expressed in the human mast cell and lung tissue (Fig. 2). The presence of significant levels of MCCEMP1 transcript in lung tissue may be attributed to the presence of peripheral mast cells. Several lines of evidence suggest that MCCEMP1 might be involved in mechanisms that regulate mast cell differentiation and/or immune responses. First, the change in expression of MCCEMP1 mRNA in mast cells appeared to be dependent on the conditions of mast cell growth and development. In the primary cord blood-derived CD34⁺ cells cultured for 1 week, the MCCEMP1 expression level was minimal. After 5 weeks, the MCCEMP1 mRNA levels reached a peak (Fig. 3). Second, the promoter region of MCCEMP1 contains NF- κ B and NF-AT binding motifs. Both transcription factors were found to regulate a wide variety of immune receptor genes [23,24]. Third, MCCEMP1 was mapped on human chromosome 19p13.3 neighboring genes related to immune responses, such as CD23, or low affinity receptor for IgE, and FIZZ3, a protein found in inflammatory zone 3 [25]. The discovery of MCCEMP1 expressed in human lung mast cells may shed new light on our understanding of mast cell regulation and the pulmonary diseases in which mast cells play pivotal role, such as asthma and chronic obstructive pulmonary disease. Further studies are required to understand the biological function of MCCEMP1 and its potential role in immune regulation and allergic diseases. The availability of MCCEMP1 cDNA, recombinant protein, and antibodies should facilitate the advancement of such studies.

Materials and methods

Cell culture

Human cord blood CD34⁺ cells (BioWhittaker) were cultured up to 9 weeks in the RPMI 1640 (Invitrogen) growth medium supplemented with 20% FBS (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine, 50 μ M 2-mercaptoethanol, 100 U/ml penicillin, 100 μ g/ml streptomycin, 10 μ g/ml gentamicin, 80 ng/ml SCF, 50 ng/ml IL-6, and 5 ng/ml IL-10. Cells were stained with anti-tryptase mAb to determine the percentage of mast cells. Cell suspensions were seeded at a density of 5×10^5 cells/ml and cytokine-supplemented medium was replaced once a week. Recombinant human IgE was used for IgE cross-linking experiments. Other cell lines were cultured following ATCC's recommendations.

cDNA subtraction and cloning

Total RNAs were isolated from CBMC and THP-1, TF-1, and Daudi cells using the RNeasy Prep system (Qiagen

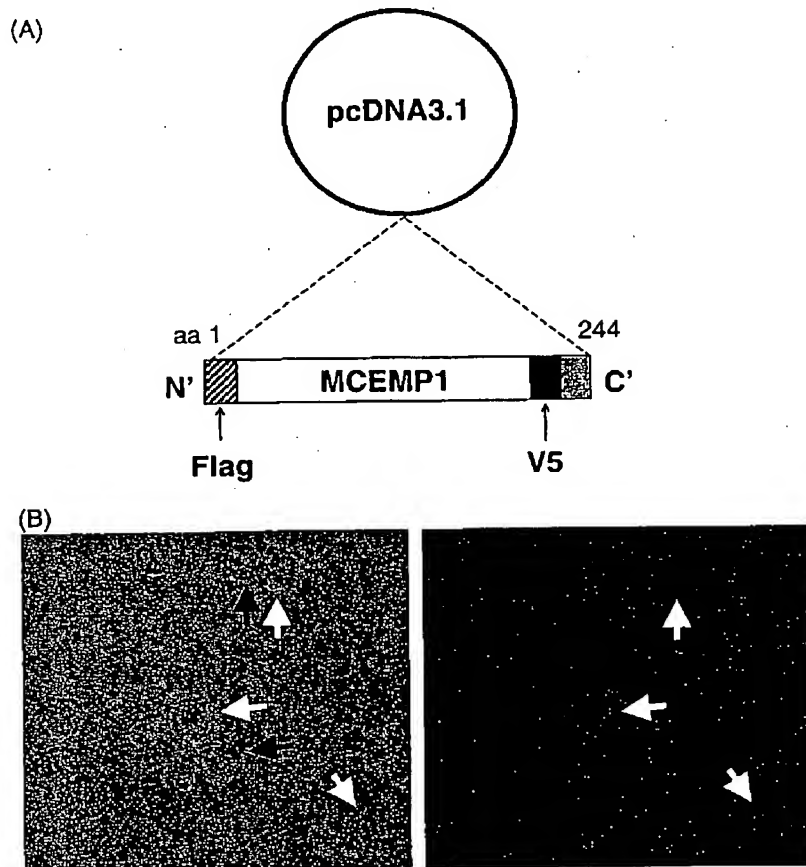


Fig. 3. (A) Expression construct MCEMP1-FV. Flag-tagged MCEMP1 was cloned in pcDNA3.1/V5-His (Invitrogen), resulting in expression construct pMCEMP1-FV. The coding region (amino acids 1–244) of the fusion protein is shown as an insert in the pcDNA3.1 vector. The open box contains the complete coding region of MCEMP1, the hatched box stands for Flag tag, the black box stands for V5 tag, and the gray box stands for His tag. (B) Immunofluorescence staining of MCEMP1-FV fusion protein in 293T cells. The cells were transiently transfected with pMCEMP1-FV and stained with FITC-conjugated anti-V5 antibody (Invitrogen) after fixation. The same microscopic view of the pMCEMP1-FV-transfected 293T cells was photographed under bright light (left) and dark field (right). White arrows indicate examples of positively stained cells, and black arrows indicate negative cells.

GmbH, Germany), and the mRNAs were isolated through an oligo(dT) push column (Stratagene, La Jolla, CA, USA). The cDNA subtraction was performed using the PCR-Select cDNA subtraction system (Clontech, Palo Alto, CA, USA) with minor modifications. Briefly, the cDNA from CBMC was used as a tester and the cDNA from a combination of THP-1 (~45%), Daudi (~35%), and TF-1 (~20%) mRNA was used as a driver in the subtractive hybridization. After two rounds of selective PCR amplification, the tagged PCR product was subcloned into plasmid vector pCR2.1 (Invitrogen) and sequenced. The cDNA sequences were BLAST-searched against GenBank database [20].

To clone the full-length MCEMP1 cDNA, two oligonucleotide primers were synthesized based on the partial sequence obtained from the subtraction and used to screen a human peripheral blood leukocyte cDNA library (OriGene). In addition, a cDNA clone was obtained from the HMC-1 cell line by RT-PCR with two oligo primers covering the start methionine codon and stop codon, respectively. The coding region of MCEMP1 cDNA was sequenced multiple times in both directions. Transmembrane domain prediction was performed utilizing hidden Markov model-based transmembrane helix recognition software [19].

RT-PCR analysis

RNAs were isolated from the following human cells: Daudi (a B lymphoblast cell line derived from Burkitt lymphoma, ATCC No. CCL-213), THP-1 (a monocytic

Table 1
MCEMP1-FV

Antibody	Anti-V5		Anti-Flag	
	Alive ^a	Fixed	Alive	Fixed
pV252-FV	+	+	–	+
pcDNA3.1	–	–	–	–

^a Cell viability was examined by trypan blue exclusion before staining with the antibodies.

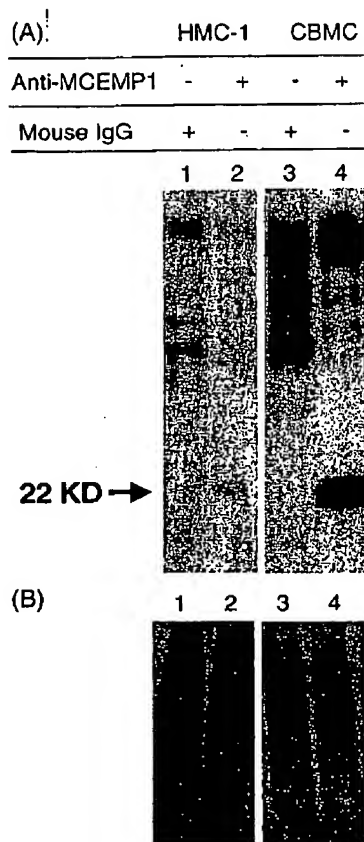


Fig. 4. (A) Immunoprecipitation of MCEMP1 from HMC-1 and CBMC by anti-MCEMP1 antibody MC1A8. Equal counts (5×10^5 cells) of HMC-1 (lanes 1 and 2) and CBMC (lanes 3 and 4) were biotinylated in each reaction, lysed in protein extraction buffer, and immunoprecipitated by MC1A8 or mouse IgG control antibody. The immunoprecipitated proteins were analyzed by Western blot using anti-biotin antibody. The 22-kDa protein matches the predicted MCEMP1 protein. (B) Coomassie blue staining of whole cell protein extract. Roughly equal amounts of protein extract from HMC-1 (lanes 1 and 2) and CBMC (lanes 3 and 4) were resolved by SDS-PAGE.

leukemia cell line, ATCC No. TIB202), TF-1 (a myeloid progenitor cell line, ATCC No. CRL-2003), HMC-1 (a mast cell line), CBMC cultured up to 9 weeks, primary lymphocytes, and peripheral blood mononuclear cells. Human buffy-coat blood samples were obtained from Gulf Coast Blood Center (Houston, TX, USA). Further cell purification process was carried out by Ficoll–Paque gradient centrifugation. Total RNA was isolated using the RNeasy Mini-Prep kit (Qiagen). The human tissue RNAs were purchased from Ambion (Austin, TX, USA).

Two sets of oligonucleotide primers (5'-TCATTCACGACCCACGAGC-3'/5'-TTGAGGTGAGGACTGTGGCATT-3' and 5'-GGAGTCCACTGGCGTCTTCAC-3'/5'-GGAGGAGTGGGTGTCGCTGTT-3') were selected from the MCEMP1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) coding region, respectively, and used in two-step RT-PCR analysis. First-strand cDNA was synthesized using reverse transcriptase, Superscriptase II, following the manufacturer's manual (Invitrogen). PCR was then performed using TaKaRa *Taq* polymerase (Sigma). Plasmid pMCEMP1-FV (Fig. 3A) was used as positive control, and molar number was calculated based on the molecular weight of pMCEMP1-FV.

Expression construct and transfection

Flag-tagged MCEMP1 cDNA was PCR-amplified by using two oligo primers (5'-CACCATGGACTACAAA-GACGATGACGACAAGGAAGTGGAGGAAATCTACAAGC and 5'-TTGAGGTGAGGACTGTGGCATT), and the PCR product was cloned into pcDNA3.1D/V5-His vector (Invitrogen) (Fig. 3A). Transient transfection was performed using the Lipofectamine Plus system (Invitrogen). Twenty micrograms of plasmid DNA was transfected into 293T cells in a 100-mm tissue culture dish, and 40 h later, the cells were harvested in PBS-based, enzyme-free cell dissociation buffer (Invitrogen) for protein analysis.

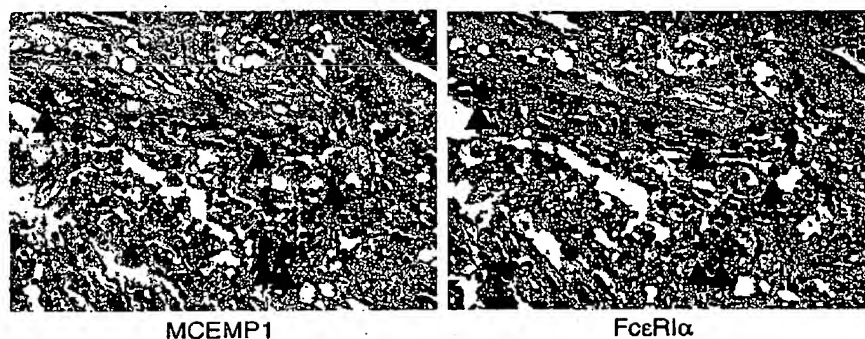


Fig. 5. Immunohistochemical staining of human lung tissue. Two consecutive sections of human lung tissue adjacent to lung tumor tissue were stained by MCEMP1 monoclonal antibody and FcεRIα antibody. Positive cells were stained in red. Arrows point to the cells stained positively both by MCEMP1 and by FcεRIα antibody. The cancer cells are on the upper right corner indicated by an asterisk.

Protein extraction and Western blot analysis

The whole cell protein sample was prepared by resuspending 3×10^5 cells in 100 μ l of ddH₂O and heated at 100°C for 5 min after adding an equal volume of 2× sample loading buffer. To separate the membrane fraction from the soluble fraction, 5×10^5 cells were subjected to lysis procedure through either homogenization or freeze–thaw cycles. For homogenization, cells were first incubated in 150 μ l of deionized water for 10 min and then passed through a No. 22 syringe needle multiple times. Thereafter a one-tenth volume of 10× lysis buffer (200 mM Tris–HCl, pH 7.6, 700 mM KCl, 50 mM EDTA) was added back and incubated for 5 min. For the freeze–thaw method, cells were suspended in 1× lysis buffer and freeze–thawed three times. Insoluble membrane fraction was separated from soluble proteins by centrifugation at maximum speed in a microcentrifuge.

The proteins were separated in a 15% SDS–PAGE. Western blot was performed as previously described [26] by using anti-Flag (Sigma) or anti-V5 mAb (Invitrogen). Protein gel, running buffer, and 2× loading buffer were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Immunofluorescence staining of transiently transfected cells

The transfected 293T cells (1×10^6) were washed and preincubated at 4°C for 20 min in 100 μ l of the enzyme-free cell dissociation buffer (Invitrogen) containing 1% BSA. Cells were then incubated with FITC-conjugated anti-Flag (20 μ g/ml) (Sigma–Aldrich) or anti-V5 mAb (10 μ g/ml) (Invitrogen) in the same buffer for 30 min. After three washes, cells were resuspended in 100 μ l of 1× PBS with 1% paraformaldehyde and analyzed by FACScan (Becton–Dickinson, Franklin Lakes, NJ, USA).

Generation of mAb against MCEMP1

The mouse monoclonal antibodies were generated against MCEMP1 by conventional hybridoma technology and screened by ELISA, FACS, and Western blot. Two of the antibodies were characterized extensively. For FACS analysis, cells were washed three times and incubated with a specific antibody. Alternatively, cells were fixed before the antibody reaction. Finally, cells were resuspended in 1× PBS with 1% paraformaldehyde for FACS analysis.

Detection of native MCEMP1 by immunoprecipitation

Cell surface proteins were biotinylated using D-biotinyl- ϵ -aminocaproic acid-*N*-hydroxysuccinimide ester following the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN, USA). Cells were then washed in Tris wash buffer (50 mM Tris–HCl, pH 7.4, 25 mM KCl, 5 mM MgCl₂, 1 mM EDTA, and 150 mM NaCl) three times

and lysed in 1× PBS with 1% Triton X-100 and protease inhibitor mix (Roche). The protein extract was incubated with the mAb against MCEMP1 and precipitated with protein A–Sepharose. After five washes in lysis buffer the protein was loaded on an SDS–PAGE gel for Western blot analysis using anti-biotin antibody. Alternatively, as a control, the whole cell protein was subjected to SDS–PAGE and Coomassie blue staining.

Immunohistochemistry of human lung tissue

Formalin-fixed and paraffin-embedded sections of human lung tissues were purchased from Spring Biosystems (Fremont, CA, USA). Each tissue specimen was deparaffinized, rehydrated, incubated for 30 min at 37°C in PBS containing 0.05% Tween 20 and 1% normal goat serum, and then incubated overnight at 4°C in buffer containing purified anti-MCEMP1 antibody. Samples were washed, incubated for 1 h at room temperature in buffer containing AP-labeled goat anti-mouse IgG, washed twice in 0.1% BSA and 0.05% Tween 20 in PBS, and then washed and incubated for 15 min at room temperature in alkaline phosphatase substrate solution. The antibody-stained tissue sections were counterstained with Gill's hematoxylin and covered with Immu-Mount (Shandon, Pittsburgh, PA, USA). Additional sections were stained with hematoxylin and eosin.

Acknowledgments

We thank Dr. Matthew Moyle and Dr. Elizabeth Fagan for reviewing and making valuable suggestions to the manuscript.

References

- [1] M.F. Gurish, K.F. Austin, The diverse roles of mast cells, *J. Exp. Med.* 194 (2001) F1–F5.
- [2] M. Welle, Development, significance, and heterogeneity of mast cells with particular regard to the mast cell-specific proteases chymase and tryptase, *J. Leukocyte Biol.* 61 (1997) 233–245.
- [3] J. Wedemeyer, S.J. Galli, Mast cell and basophils in acquired immunity, *Br. Med. Bull.* 56 (2000) 936–955.
- [4] W.-P. Fung-Leung, J.D. Sousa-Hitzler, A. Ishaque, L. Zhou, J. Pang, K. Ngo, J.A. Panakos, E. Chourmouzis, F.-T. Liu, C.Y. Lau, Transgenic mice expressing the human high-affinity immunoglobulin (Ig) E receptor α chain respond to human IgE in mast cell degranulation and in allergic reaction, *J. Exp. Med.* 183 (1996) 49–56.
- [5] I.G. Reischl, W.R. Coward, M.K. Church, Molecular consequences of human mast cell activation following immunoglobulin E-high-affinity immunoglobulin E receptor (IgE–Fc ϵ RI) interaction, *Biochem. Pharmacol.* 58 (1999) 1841–1850.
- [6] A.P. Prodeus, X. Zhou, M. Maurer, S.J. Galli, M.C. Carroll, Impaired mast cell-dependent natural immunity in complement C3-deficient mice, *Nature* 390 (1997) 172–175.
- [7] P.H. Hurt, Regulation of the inflammatory response in asthma by mast cell products, *Immunol. Cell Biol.* 79 (2001) 149–153.

- [8] P.E. Christie, W.R. Herder, Lipid inflammatory mediators: leukotrienes, prostaglandins, platelet-activating factor, in: B. Zweiman, L.B. Schwartz (Eds.), *Inflammatory Mechanisms in Allergic Diseases*, Dekker, New York, 2001, pp. 233–254.
- [9] R. Alam, G. Kampen, Cytokines in allergic inflammation, in: B. Zweiman, L.B. Schwartz (Eds.), *Inflammatory Mechanisms in Allergic Diseases*, Dekker, New York, 2001, pp. 255–274.
- [10] C. Lock, G. Hermans, R. Pedotti, A. Brendolan, E. Schadt, H. Garren, A. Langer-Gould, S. Strober, B. Cannella, J. Allard, P. Klonowski, A. Austin, N. Lad, N. Kaminski, S.J. Galli, J.R. Oksenberg, C.S. Raine, R. Heller, L. Steinman, Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis, *Nat. Med.* 8 (2002) 500–508.
- [11] V.H. Secor, W.E. Secor, C.-A. Gutekunst, M.A. Brown, Mast cells are essential for early onset and severe disease in a murine model of multiple sclerosis, *J. Exp. Med.* 191 (2000) 813–822.
- [12] W.R. Macon, M.C. Kinney, A.D. Glick, R.D. Collins, Marrow mast cell hyperplasia in hairy cell leukemia, *Mod. Pathol.* 6 (1993) 695–698.
- [13] R.M. Tuder, A pathologist's approach to interstitial lung diseases, *Curr. Opin. Pulmon. Med.* 2 (1996) 357–363.
- [14] C. Benoist, D. Mathis, Mast cell in autoimmune diseases, *Nature* 420 (2002) 875–878.
- [15] D.E. Woolley, L.C. Tetlow, Mast cell activation and its relation to proinflammatory cytokine production in the rheumatoid lesion, *Arthritis Res.* 2 (2000) 65–74.
- [16] A.M. Dvorak, New aspects of mast cell biology, *Int. Arch. Allergy Immunol.* 114 (1997) 1–9.
- [17] V. Patella, G. de Crescenzo, B. Lamparter-Schummert, G. De Rosa, M. Adt, G. Marone, Increased cardiac mast cell density and mediator release in patients with dilated cardiomyopathy, *Inflammation Res.* 46 (Suppl. 1) (1997) S31–S32.
- [18] M. Kozak, An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs, *Nucleic Acid Res.* 115 (1987) 8125–8148.
- [19] A. Krogh, B. Larsson, G. von Heijne, E.L.L. Sonnhammer, Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes, *J. Mol. Biol.* 305 (2001) 567–580.
- [20] S.F. Altschul, T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, *Nucleic Acids Res.* 25 (1997) 3389–3402.
- [21] P. Bucher, Weight matrix descriptions of four eukaryotic RNA polymerase II promoter elements derived from 502 unrelated promoter sequences, *J. Mol. Biol.* 212 (1990) 563–578.
- [22] R.S. Selvan, J.H. Buitterfield, M.S. Krangel, Expression of multiple chemokine genes by a human mast cell leukemia, *J. Biol. Chem.* 269 (1994) 13893–13898.
- [23] V. Dixit, T.W. Mak, NF- κ B signaling: many roads lead to Madrid, *Cell* 111 (2002) 615–619.
- [24] G.R. Crabtree, E.N. Olson, NFAT signaling: choreographing the social lives of cells, *Cell* 109 (2002) S67–S79.
- [25] I.N. Holcomb, R.C. Kabakoff, B. Chan, T.W. Baker, A. Gurney, W. Henzel, C. Nelson, H.B. Lowman, B.D. Wright, N.J. Skelton, G.D. Frantz, D.B. Tumas, F.V. Peale Jr., D.L. Shelton, C.C. Hebert, FIZZ1, a novel cysteine-rich secreted protein associated with pulmonary inflammation, defines a new gene family, *EMBO J.* 19 (2000) 4046–4055.
- [26] K. Li, Y. Li, J.M. Shelton, J.A. Richardson, E. Spencer, Z.J. Chen, X. Wang, R.S. Williams, Cytochrome c deficiency causes embryonic lethality and attenuates stress-induced apoptosis, *Cell* 101 (2000) 389–399.